Optimization of universal Chrome Azurol S (CAS) siderophore detecting media with an economical dye substitute using *Pseudomonas fluorescens* as a test organism

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Abstract

Siderophores are iron sequestering molecules of microbial origin. The present study evaluated the use of alternative azo dyes to the expensive Chrome Azurol S (CAS) dye incorporated in siderophore detecting media. Pseudomonas fluorescens isolated from garden soil by method of serial dilution was identified as a potent producer of hydroxamate type siderophore, based on the results of Tetrazolium test. Methyl orange (MO) and Eriochrome Black T (EBT), chosen as test dyes for the study, were assessed at increasing (20ppm, 30ppm, 40ppm and 60ppm) concentrations to yield a colour change indicative of positive production of siderophores. It was observed that all concentrations of MO modified media proved detrimental to the growth of Pseudomonas, possibility of a dye intolerance or dye toxicity. The modified media incorporated with 20ppm and 30ppm EBT exhibited a colour change from red to blue after 48 hours of incubation.

Higher concentrations of EBT, 40ppm and 60ppm inhibited the growth of Pseudomonas. A standard siderophore Desferrioxamine was spotted on every modified media as a positive control. The concentrations of 20ppm and 30 ppm EBT were further formulated in liquid broth to ease the process of identifying siderophore-producing organisms with a simple inoculation. The expected colour change positive for siderophore production (red to blue) was significant in 30ppm EBT incorporated modified broth and insignificant in 20ppm EBT incorporated modified broth.

Thus, in the present study, we have been successfully able to identify EBT as a competitive alternative to CAS dye in identification of siderophore-producing bacteria for a mixed community of organism, which is more economical.

Keywords: Siderophores, Siderophore producing bacteria, Chrome Azurol S (CAS), Eriochrome Black-T, *Pseudomonas fluorescens*, Tetrazolium test, Hydroxamate.

Introduction

Microorganisms utilise iron to fuel critical biological processes of oxidation, replication, respiration and photosynthesis. More or less, iron plays an integral role in the function of an enzyme, mainly serving as an essential metal cofactor. However, the concentrations of iron in the rhizosphere are limited, especially in neutral and alkaline soils. This encourages competition between microbial colonies in the soil for a limiting metal ion. Besides, the solubility of ferric ions under physiological pH values is drastically low, owing to their complete hydrolysis. Thus, to circumvent this iron stress, microorganisms have developed a strategy to chelate and solubilise iron from the environment by production and secretion of low molecular proteins, termed siderophores [2, 8].

Siderophores are low molecular weight iron-chelating proteins produced by bacteria, fungi, actinomycetes and certain algae under iron-limiting conditions[12]. The primary role of siderophores is to scavenge iron from the environment and make it available to microbial cells. These iron chelators bear side chains and functional groups as high-affinity ligands to ferric ions in the soil. Upon successful coordination, the iron is transported within the cell through the cell membrane. Therefore, siderophores are also referred to as 'Microbial magnets', fetching iron from the environment. Siderophores are classified into carboxylate, hydroxamates, catecholates and mixed types, based on their structure, functional group and types of ligand[1].

Siderophores have found numerous applications in agricultural, medical and environmental sciences. Siderophores, fetch iron from the environment and thus can promote the growth of several unculturable microorganisms, aiding microbiologists and pathologists in the study of infectious diseases. The production of siderophores by a member of a microbial community selectively encourages the growth of particular microbes thus serving a significant role in ecology. In the field of agriculture, the incorporation of suitable siderophores in the soil can enhance plant growth by increasing iron uptake in plants.

Siderophores can in used in the remediation of soil and water. Soil and water contaminated with heavy metal can be treated and detoxified by siderophores. Siderophores can aid in the detection of iron in different environments, emphasizing its role as a biosensor. In the medical field, to

combat drug-resistant bacterial species, siderophore molecules have been used as carriers of antibiotics inside bacterial cell [9].

The applications served by siderophores have prompted for its synthesis on a large scale, which can be met either chemically or biologically. Chemically synthesized siderophores mimic their biological relatives in function. Biologically, these siderophores can be harvested from culture broths of bacteria, fungi and algae. Bulk production of siderophores can be carried out in iron limiting culture media broths, inoculated with a potent siderophore-producing organism. The universal method for detecting siderophore production by an organism is by isolation on a media supplemented with Chrome Azurol S (CAS) and hexadecyltrimethylammonium bromide (HDTMA) as indicators[12]. This isolation assay is based on competition for iron between the ferric complex of an indicator dye, CAS and a chelator or siderophore produced by the test microorganism.

The CAS/HDTMA binds non-covalently with ferric iron to produce a blue colour in the absence of siderophore. When a stronger chelator such as a siderophore is produced, iron is stripped off the CAS/HDTMA complex by the siderophore, resulting in colour change mostly from blue to orange [6].

However, the manufacturing process of CAS dye is extremely expensive and its disposal is another environmental issue. It would become highly lucrative if an economical alternative dye is identified and used in the screening of siderophores. Such a substitute must be able to indicate a significant colour in iron-bound and iron-unbound states. The present study aims to evaluate the use of such an alternative Eriochrome Black T (EBT) as an economical replacement to CAS.

Eriochrome Black T is a hydroxyl-aryl azo dye, soluble in water and alcohol. It functions as an acid-base indicator in titration reactions. EBT unusually forms a complex with Fe^{3+}, Ca^{2+} and Mg^{2+} and a few other metal ions. In its protonated (metal bound) form, EBT is red in colour and turns blue when stripped off of the metal, now in deprotonated (metal unbound) form.

Therefore, in theory, when ferric ions bind to EBT (protonated form) in the media, they will develop a red colour. When an inoculated bacterium produces siderophores in this media, the siderophore will scavenge iron from the EBT-Fe complex indicated by a colour change from red to blue [3].

Pyoverdines (PVD's) are the main type of siderophores secreted by *Pseudomonas fluorescence* in an iron-limiting environment. *Pseudomonas fluorescence* belongs to the fluorescent pseudomonads group, a gram-negative soil bacterium. It can be isolated easily from garden soil and produces large amounts of PVD. This PVD aids *Pseudomonas* in competition for iron in the soil. Therefore, due to the ease of isolation and efficient production of PVD, *Pseudomonas* was chosen as the test organism in the study.

Thus, the present investigation addresses the economic concerns for the identification of siderophore-producing bacteria and biological production of siderophore from bacteria and has therefore attempted to optimise the existing isolation media for siderophore-producing bacteria with an efficiently identical substitute.

Material and Methods

Sampling Location and Sample Collection: 0.5g rhizospheric soil samples were collected from four different locations selected randomly in Mumbai, for isolation of siderophore-producing *Pseudomonads*. The soils were collected from Garden area (Oval maidan, Churchgate, Mumbai), Forest area (BARC, Trombay, Mumbai), Petrol pump (V.P road, Charni road, Mumbai) and a public park (Hanging garden, Mumbai). Soil samples were collected in sterile containers and transported to the laboratory within 24 hours.

Isolation of *Pseudomonas fluorescens* from soil: Isolation of *Pseudomonas fluorescens* from five soil samples was carried out by serial dilution and plating method. 0.5g soil from each of the five sample was serially diluted in saline up to 10^{-6} dilution. The last three dilutions were spread plated on Cetrimide agar, King's B agar and Nutrient agar, out of which King's B media is an iron deficient media. After incubation at 37 °C for 24 hours, growth was observed for all dilutions (decreasing colony count with increasing dilution) in all three media plates. Incubated King's B media plates were exposed to the UV- Transilluminator at 365nm for a few seconds, to select and identify colonies producing diffusible fluorescent pigment. Three colonies (isolate A, B, C) from 10^{-6} dilution of forest soil emitted fluorescence; each were purified and maintained on King's B agar slant at 4°C[11].

Biochemical characterisation and 16S rRNA sequencing for identification of the bacterial isolates: Each of the three isolates was assessed though a series of biochemical test for primary identification according to Bergey's Manual of Determinative Bacteriology. The biochemical tests, Indole test, Methyl red test, Voges Proskauer test, Citrate test, Catalase test, oxidase test and KOH tests were carried out, in addition to gram staining, to distinguish the culture from *Pseudomonas aeruginosa*. On the basis of morphological, cultural and biochemical characteristics, isolate B was identified as *Pseudomonas fluorescens* [5].

To affirm culture identity, a pure colony was outsourced at Triyat Genomics, Nagpur, Maharashtra, for 16S rRNA partial sequencing for culture identification. DNA was isolated from the culture; its quality was evaluated on 1.0% agarose gel on which a single band of high-molecular weight DNA was observed. Fragment of the gene was amplified by

PCR and a single discrete PCR amplicon band was observed when resolved on agarose gel. The PCR amplicon was purified by column purification to remove contaminants. DNA sequencing reaction of PCR amplicon was carried out with 27F primer using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyser.

The gene sequence obtained was compared with the NCBI Genbank database by a nucleotide BLAST. Based on maximum identity score, first ten sequences were selected and aligned using multiple alignment software programs.

Preparation of standard CAS media and modified EBT/MO media: Before beginning formulation, all glasswares were soaked overnight in 6M HCl and rinsed with double distilled water several times to remove iron traces. Solution 1 was prepared by dissolving 0.06g of CAS in 50ml of double distilled water. Solution 2 was prepared by dissolving 0.0027g of $FeCl_3.6H_2O$ in 10ml of 10mM HCl. Solution 3 was prepared by dissolving 0.073g of HDTMA in 40ml of ddH_2O. Solutions 1, 2 and 3 were mixed to prepare CAS dye reagent and autoclaved [6]. To prepare modified EBT/MO dye reagent, solution 1 containing 0.02g, 0.03g, 0.04g and 0.06g EBT/MO was added to obtain 20ppm, 30ppm, 40ppm and 60ppm concentrations respectively. Solution 2 and Solution 3 were same as that for CAS dye reagent (Fig. 1).

For preparing standard and modified media, 200ml of cetrimide broth formulated with 1g alum oxide (to remove all the trace elements) was prepared and autoclaved. After autoclaving, the broth was filtered through Whatmann filter paper no. 1 followed by addition of agar and then autoclaved. 20ml of autoclaved CAS dye reagent and EBT/MO dye reagent were added aseptically in each 200ml autoclaved cetrimide agar and mixed thoroughly. After mixing, CAS, EBT and MO agar plates were prepared (Fig. 1)[6,12]. To prepare liquid media broth, same protocol was followed, except no agar was introduced after filtration of media.

Assessment of modified EBT and MO media to detect siderophore production: The screening was performed by spot inoculation of isolated *Pseudomonas fluorescens* onto a chrome azurol S (CAS) agar and modified EBT/MO agar plates. The plates were incubated 18–24 hrs at 28 °C. Few other laboratory strains, *Escherichia coli, Staphylococcus aureus, Corynebacterium diphtheria* and *Pseudomonas aeruginosa, Salmonella typhi,* were also spotted on standard CAS plates and modified EBT/MO plates. This step was undertaken to compare production of siderophores between *Pseudomonas fluorescens* and other organisms and the ability to the media to detect the same.

The basic principle underlying this test was that if siderophores are produced and secreted in the media, iron would be scavenged from the iron-dye complex leading to the formation of iron siderophore complex. This will be accompanied with a colour change from red to blue by the free dye. Desferrioxamine was used as a standard and spotted alongside every test culture [4]. The modified EBT/MO media broths and standard CAS dye media broth were prepared in three sets – a positive control with desferrioxamine, a negative media control and a test with a loopful of culture suspension of *Pseudomonas fluorescens* (0.1 O.D at 545nm) inoculated.

Characterization of siderophores: A change in colour from blue to orange in CAS assay indicated hydroxamate type siderophore[10]. The tetrazolium salt test was performed to confirm the chemical nature of siderophore being produced by *Pseudomonas fluorescens*. This test takes advantage of the ability of hydroxamic acids to reduce tetrazolium salt by hydrolysis of hydroxamate groups in the presence of a strong alkali producing red colour. To 1ml of cell-free culture extract, a pinch of Tetrazolium salt and 2 drops of 2N NaOH were added. Instant appearance of a deep red colour indicated hydroxamate nature of siderophore [13].

Results and Discussion

The increase in the applications and knowledge of siderophore function has ignited interest in the research community.

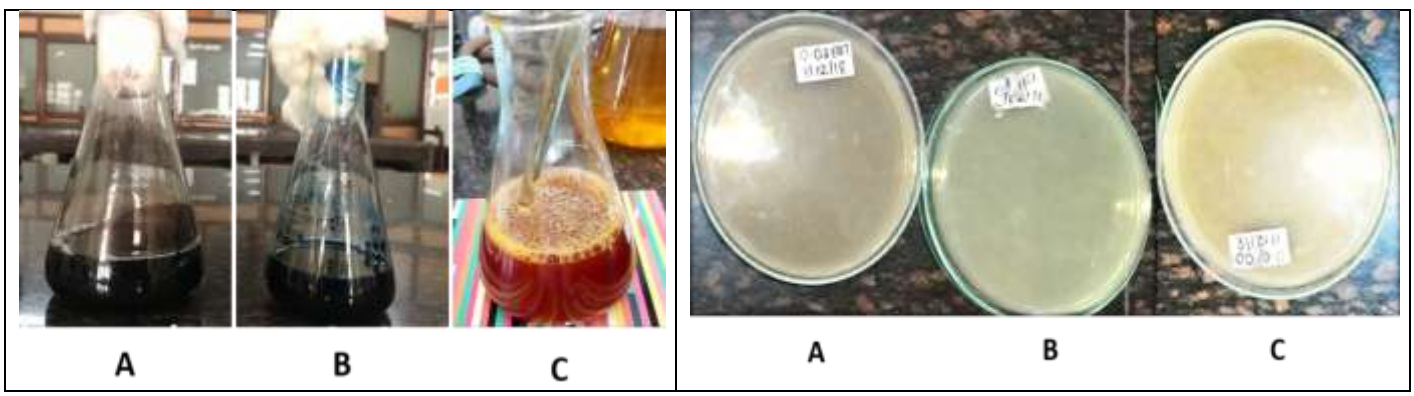

Fig. 1: *Left* – Preparation of (A) Eriochrome Black T, (B) CAS and (C) Methyl Orange dye reagents of 60ppm concentration. Similarly, dye reagents were prepared at 20ppm, 30ppm and 40ppm for EBT and MO. *Right* – Prepared media plates of (A) EBT, (B) CAS and (C) MO

The emergence of newer plant diseases and plant pathogens have also attracted the agricultural sciences to provide measures to overcome crop destruction. The importance of siderophores in bioremediation has eliminated the risk of opportunistic pathogens being introduced as microbial remediators.

The harvest and industrial production of siderophores begins in the laboratory with a search for an efficient siderophore-producing organism. Isolation and selection of such an effective siderophore secretor must therefore be made easy, economical and successful. The outcome of this study has served the purpose of improvising an existing and expensive media with a much resourceful and affordable dye reagent, comparable in results.

Isolation of *Pseudomonas fluorescens*: The isolation of *Pseudomonas fluorescens* was carried out from five different samples on cetrimide media, King's B media and nutrient agar by method of serial dilution. Colonies in decreasing numbers were observed on all dilution plates (10^{-6}, 10^{-5} and 10^{-4}) for every sample. For detection, plates were exposed to UV Transilluminator, plates spread with diluted forest soil showed fluorescent colonies for forest sample (Fig 2). Three colonies (arrow marked in fig. 2) were purified and processed for biochemical test and 16S rRNA sequencing identification.

Biochemical characterisation and 16S rRNA identification of isolate: The three isolates (A, B, C) were characterised for primary identification based on biochemical tests. Table 1 enlists the tests performed and result obtained for each isolate along with results of standard *Pseudomonas fluorescens* (Fig. 3) and *Pseudomonas aeruginosa*.

On comparing the biochemical test chart of the two standard organisms with the isolates, it was observed that isolate B gave the identical tests results to *P. fluorescens* (Fig. 3 and Table 1). Therefore, a pure colony of isolate B was chosen and sent for 16S rRNA sequencing for identification (Fig. 4 shows the sequence retrieved). Based on the results generated from BLAST (Alignment shown in fig. 4) and multiple sequence alignment, it was confirmed that the isolated organism was *P. fluorescens* showing 97% identity with *P. fluorescens* strain NBRC 14160 16S rRNA.

Fig. 2: Fluorescent colonies of *Pseudomonas spp* (isolate A, B and C indicated with black arrow) observed under a UV- Transilluminator after on King's media

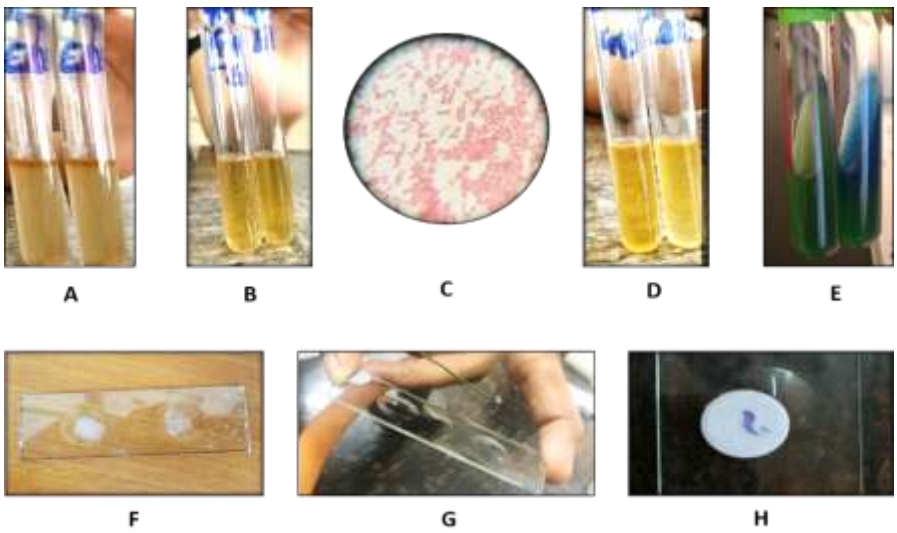

Fig. 3: Biochemical tests conducted for primary identification of *Pseudomonas fluorescens; A – Negative Indole test, B – Negative MR test, C – Gram negative coccobacilli observed under 100X oil immersion lens, D – Negative VP test, E – Positive citrate test, F – Positive catalase test, G – Positive KOH test (a slimy string is observed given only by Pseudomonas fluorescens* and not by *Pseudomonas aeruginosa, H – Positive catalase test (In A,B,D,E, left are control tubes and right are test tubes)*

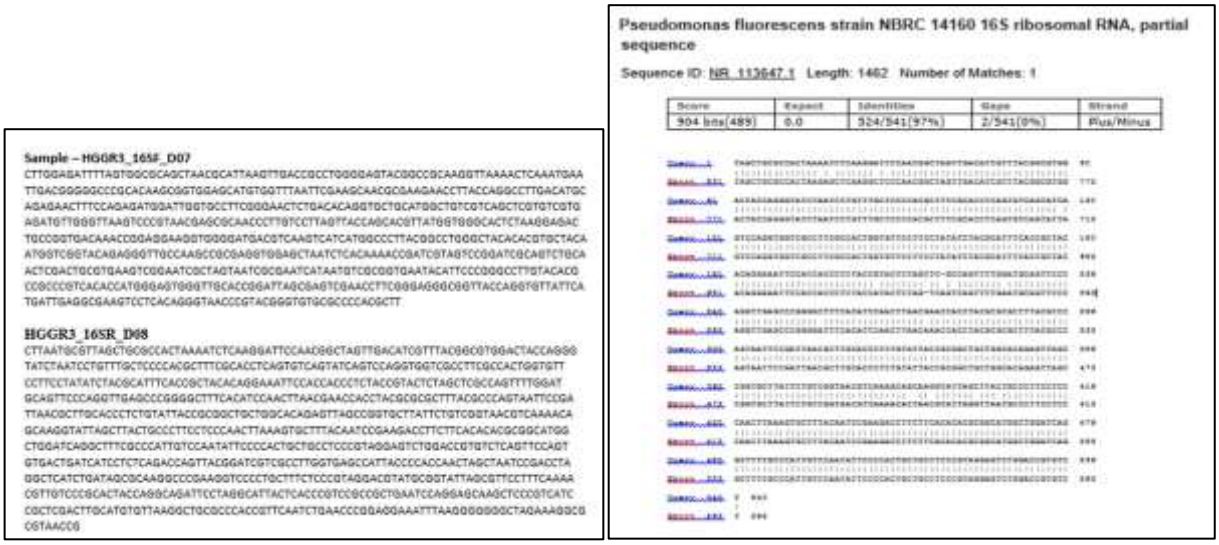

Fig. 4: Above is the image of the partial sequence of 16S rRNA gene (forward – top, reverse – bottom). To the right is the best alignment obtained by BLAST, of the query sequence and GenBank entry of *P. fluorescens* strain NBRC 14160 16S rRNA showing 97% identity

Table 1
Biochemical test results obtained for the three isolates. On comparing the standard biochemical chart of *P. fluorescens* with the three isolates, it was found that isolate B was most probably *P. fluorescens*

TEST	Indole	Methyl red (MR)	Vosges Proskauer (VP)	Citrate	Catalase	Oxidase	KOH Test	Gram Staining
Isolate A	Negative	Negative	Negative	Positive	Positive	Positive	Negative	Negative
Isolate B	Negative	Negative	Negative	Positive	Positive	Positive	Positive	Negative
Isolate C	Negative	Negative	Negative	Positive	Positive	Positive	Negative	Negative
P. fluorescens	Negative	Negative	Negative	Positive	Positive	Positive	Positive	Negative
P. aeruginosa	Negative	Negative	Negative	Positive	Positive	Positive	Negative	Negative

Screening of modified EBT and MO media: The modified EBT and MO solid media was spot inoculated with the identified test culture, *P. fluorescens*. No growth was observed on MO incorporated media plates, at every concentration and it was suggested that MO was highly toxic to *Pseudomonas* even at minimalistic concentrations and/or MO was incapable of forming an iron-dye complex and yielding a positive colour change.

No colonies were observed for the other five microorganisms spot inoculated along with *P. fluorescens*, indicating immediate and dangerous dye toxicity. The inability of MO to show the classic iron-dye colour change reaction was further justified by no colour change observed for the standard Desferrioxamine. Therefore, further evaluation of media competency was conducted on EBT modified media.

Growth in the form of visible colonies at the spot inoculation site was observed for low concentrations of EBT, 20ppm and 30ppm (Fig. 6). However, no growth was observed at spot inoculation sites on high concentrations of EBT incorporated media plates, 40ppm and 60ppm (Fig. 6). The periphery of *Pseudomonas fluorescens* colonies obtained on 20ppm and 30ppm modified EBT media did exhibit the expected color change from red to blue (Fig 6 yellow arrow). Both the concentrations were able to yield the classic iron-dye color change reaction, indicating the success of the experiment and completion of the experimental objective.

Other organisms spotted on the same plates also showed colony formation, indicating that EBT was a less toxic dye and concentrations of 20ppm and 30ppm were tolerant enough for satisfactory growth of microorganisms. The color change on both plates (20ppm and 30ppm) was observed after 24 hrs of incubation at room temperature. The plates were further incubated for another day to assess whether the color change would develop strongly and grow more visible. After 48 hrs incubation, the color change was prominent demanding a longer incubation period. The results signified that EBT also demonstrated a colour change because of electron transfer, a mechanism similar to CAS dye. EBT is red in colour when protonated (iron bound). However, it turns blue in colour when it undergoes deprotonation (iron-unbound).

On the standard CAS dye plate, the colony of *Pseudomonas fluorescens* was surrounded by orange colour, justifying the colour change from blue to orange (Fig. 5) due to siderophore production and secretion into the media.

Standard desferrioxamine solution gave an immediate colour change when applied on CAS (Fig. 5) and EBT (Fig. 6 red arrow) media plates. Although colour development was observed on both plates (20ppm and 30ppm), the colour was enhanced, brighter and very evident in 30ppm modified EBT agar plate. Therefore, 30ppm EBT concentration was chosen to be evaluated at the media broth level.

The standard CAS dye media broth was able to show colour change (Fig. 7a) after 24 hrs incubation at room temperature and the modified EBT media broth was also able to show colour change from red to blue (Fig. 7b).

The results obtained after 24 hrs were conclusive to detect a siderophore-producing organism. Standard desferrioxamine gave immediate colour change in CAS media and 2-3 hrs delayed colour change in modified EBT media. The negative media controls both EBT and CAS, exhibited no colour change demonstrating that the test reaction only showed colour due to siderophore production.

Characterization of siderophore produced by *Pseudomonas fluorescens*: The culture was allowed to grow in 50ml cetrimide broth for 24 hrs at 37 °C. After 24 hrs, the broth was filtered to separate media and culture. When the cell free media extract was tested for hydroxamate test, a red colour was observed immediately (Fig. 8). This colour change was an affirmation of hydroxamate type of siderophores produced by *Pseudomonas fluorescens*.

Conclusion

In the present pilot study, the isolated *Pseudomonas fluorescens* was found to be a hydroxamate type siderophore producer. CAS assay is a gold standard adopted worldwide for the identification of the siderophore-producing organism, but CAS is expensive and therefore only accessible to well financed laboratories. Thus, the requirement for a substitute dye arises, which is not only affordable but also competitive to CAS.

EBT, a common titration indicator was chosen for the study, to optimise the CAS media with a reasonable and efficient dye. When the local rates for CAS and EBT were compared, it was found that 1g CAS dye cost Rs. 2000, whereas 1g EBT dye cost only Rs. 32. This was a very significant difference in pricing, yet a marginal difference in efficiency. Also, 60ppm concentration of CAS was minimum to obtain an evident colour change for siderophore production, whereas only 30ppm EBT concentration was sufficient to indicate colour change for siderophore production.

Fig. 5: 60ppm CAS media plates showing a blue to orange colour change indicating siderophore production, circled in white is the standard and circled in yellow is the spot inoculation site of *P. fluorescens*

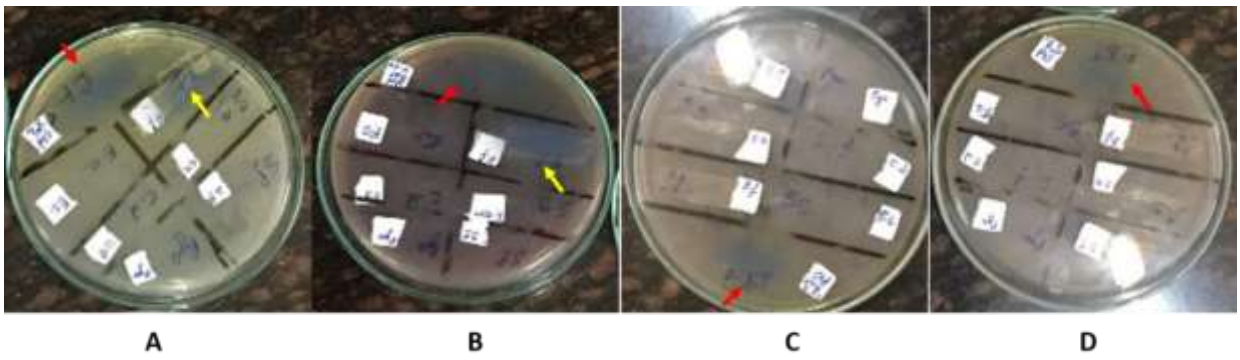

Fig. 6: EBT media plates (A – 20ppm, B – 30ppm, C – 40ppm, D – 60ppm) spot inoculated with standard, *P. fluorescens and* other test organisms. Red to blue color change for standard is visually evident in all plated indicated by red arrow. Red to blue color change for *P. fluorescens* is visually evident in plates A and B indicated by yellow arrow. No growth was observed on plates C (40ppm) and D (60ppm)

Fig. 7(a): Standard CAS broth with 60ppm concentration. Media control is in the centre, on the left is the standard, which has changed colour form blue to orange and to the right is the test inoculated with *P. fluorescens* showing colour change form blue to orange because of siderophore production.

Fig. 7(b): Modified EBT broth with 30ppm concentration. Media control is on the left, to the right is the standard, which has changed colour from red to blue and in the centre is the test inoculated with *P. fluorescens* showing colour change from red to blue because of siderophore production.

Fig. 8: Development of red colour in tetrazolium test affirming hydroxamate nature of siderophores produced by *P. fluorescens.*

Due to limited laboratory funding, the authors could not avail PIPES buffer and CAS amino acids crucial to the operation of the media. Thus, the delayed colour change observed on solid EBT modified media (24hrs) could be attributed to the absence of buffering agents and stabilisers in the media. Therefore, the future of this raw study targets the formulation of modified media with buffer and cas amino acids, to enhance colour change and overcome time delay.

The authors also plan to escalate the study by evaluating the stability of the new media and this assay at different temperatures and with different organisms. Fungi and algae too, are known to produce siderophores, so gentle tweaking in media composition and ingredients may allow for the screening of fungal and algal siderophore producers.

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